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Reoto Tani, Wataru Kawamura, Tetsuro Morita, Christophe Klopp, Marine Milhes, Yann Guiguen, Goro Yoshizaki, Ryosuke Yazawa

Fisheries Science (2021) 87:785–793
https://doi.org/10.1007/s12562-021-01548-z

ORIGINAL ARTICLE

Biology

Development of a polymerase chain reaction (PCR)-based genetic sex identification method in the chub mackerel *Scomber japonicus* and blue mackerel *S. australasicus*

Reoto Tani[1] · Wataru Kawamura[1] · Tetsuro Morita[2] · Christophe Klopp[3] · Marine Milhes[4] · Yann Guiguen[5] · Goro Yoshizaki[1,2] · Ryosuke Yazawa[1]

Received: 3 June 2021 / Accepted: 16 August 2021 / Published online: 30 August 2021

Abstract

Chub mackerel *Scomber japonicus* and blue mackerel *S. australasicus* have become important aquaculture target species in Japan. For an efficient and stable aquaculture production of mackerels, it is desirable to work with a female-biased broodstock and thus to be able to manage precisely their sex ratio. As these two mackerel species do not have any external sexual dimorphism, direct biopsy of the gonads or measurement of sex hormone plasma levels is used in research to determine their phenotypic sex. However, these methods are associated with handling stress that results in gonadal atresia. Besides, it is difficult to identify sex using these methods in immature individuals. Therefore, genetic sexing would be desirable in these species to overcome these problems. In this study, we developed molecular sex identification tools for both chub and blue mackerels using sex-specific single-nucleotide variations (SNVs) identified by pool-sequencing (pool-seq) analyses. Genomic DNA was isolated from 30 males and females, in each species, and sequenced as pooled samples. Results of these pool-seq analyses identified a clear XX/XY male heterogametic sex-determination system in blue mackerel and a ZZ/ZW female heterogametic system in chub mackerel. Using this pool-seq information, we designed primer sets for genetic sex identification in both species. Results demonstrated that these primer sets are robust and accurate in assessing the genotypic sex of both blue and chub mackerels (accuracy > 99.5% in the chub, and > 94.5% in the blue mackerel) in various geographical samples of different ages. This method can now be applied for the efficient management of sex ratios in both mackerel aquaculture populations and wild-caught animals for fisheries management.

Keywords Chub mackerel *Scomber japonicus* · Blue mackerel *S. australasicus* · Molecular sexing · Broodstock management · Pool-seq

Introduction

Chub mackerel *Scomber japonicus* and blue mackerel *S. australasicus* are distributed in Japan's coastal waters. Both species are major targets for commercial fisheries in Japan and have been selected as aquaculture targets recently (Mendiola et al. 2008; Hashimoto et al. 2019). Hatchery-reared breeding of mackerels has also been established recently, and commercialization of artificial seedlings has started in Japan (Matsuyama et al. 2005; Murata et al. 2005; Shiraishi et al. 2005). However, gamete collection in mackerels remains difficult due to the uncertainty of maturation in captivities (Amezawa et al. 2018). This difficulty is mainly due to the frequent occurrence of female mackerels

✉ Ryosuke Yazawa
ryazawa@kaiyodai.ac.jp

1 Department of Marine Biosciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan Minato-ku, Tokyo 108–8477, Japan

2 Institute for Reproductive Biotechnology for Aquatic Species, Tokyo University of Marine Science and Technology, 4-5-7 Konan Minato-ku, Tokyo 108–8477, Japan

3 INRAE, Sigenae, Genotoul Bioinfo, Toulouse, France

4 GeT-PlaGe, INRAE, Genotoul, Castanet-Tolosan, France

5 INRAE, LPGP, Rennes, France

whose maturation is retarded due to physiological stress in the rearing environment. To overcome this problem, two approaches can be explored. The first one is to develop a better method to induce female final maturation in mackerel, and several efficient maturation induction methods have been developed recently (Amezawa et al. 2018; Ohga et al. 2020). The second approach is to work with an increased number of females that could be used as broodstock candidates. However, identifying the sex of these two *Scomber* species with morphological characteristic is impossible as they do not possess any sexual dimorphism in their external appearance (Kobayashi et al. 2011; Amezawa et al. 2018). Techniques such as direct biopsy of the gonads by catheterization or measurement of sex hormone plasma levels have therefore been used to determine sex in these two mackerel species (Amezawa et al. 2018). However, mackerels have a low tolerance for such invasive samplings, and the handling stress induced by these manipulations occasionally results in ovarian atresia. Therefore, there is a need for minimally invasive approaches allowing the identification of females in aquaculture.

One of the less invasive techniques of sex determination is the genetic testing method. This method detects sex differences within sex-determining regions of sex chromosomes. Because these genetic tests can be performed using small fin-clip samples or skin mucus swiping, stress can be reduced to a minimum during sample collection. Previously, various molecular marker techniques, such as amplified fragment length polymorphisms, random amplified polymorphic DNAs (RAPDs), and simple sequence repeats (SSR), have been used to identify sex-linked molecular markers in fish. By employing these sex-linked DNA markers, sex identification methods have been developed in various species such as the African catfish *Clarias gariepinus* (Kovács et al., 2000), rainbow trout *Oncorhynchus mykiss* (Felip et al. 2005), half-smooth tongue sole *Cynoglossus semilaevis* (Chen et al. 2007), yellowtail *Seriola quinqueradiata* (Fuji et al. 2010), Nile tilapia *Oreochromis niloticus* (Lee et al. 2011), Pacific halibut *Hippoglossus stenolepis* (Galindo et al. 2011), barred knifejaw *Oplegnathus fasciatus* (Xu et al. 2013), and tiger puffer *Takifugu rubripes* (Matsunaga et al. 2014). With the advent of novel next-generation sequencing methods, many new approaches have been recently developed to identify sex-specific markers, and sex-specific markers have been isolated from various species, such as rockfishes *Sebastes carnatus* and *Sebastes chrysomelas* (Fowler and Buonaccorsi 2016), Pacific bluefin tuna *Thunnus orientalis* (Suda et al. 2019), and spotted knifejaw *Oplegnathus punctatus* (Li et al. 2020). However, for non-model species such as mackerels, the construction of high-resolution genetic linkage maps or reference genome sequences is still expensive and time-consuming; therefore, a more practical method is required. Alternatively, genome sequencing of pools of individuals provides genome-wide polymorphism data at a considerably lower cost than sequencing many individual genomes (Schlötterer et al. 2014). This technique is considered an easy and cost-saving approach to identify sex-linked DNA markers, such as single-nucleotide variations (SNVs) in non-classical model species.

Therefore, we applied this approach to identify sex-specific SNVs in chub and blue mackerels and used this information to develop a polymerase chain reaction (PCR)-based method for genetic sex identification in these two species.

Materials and methods

Sample fish and DNA extraction

In November 2018, a set net was used to catch chub and blue mackerel in Tateyama Bay, Chiba, Japan. A total of 120 fishes were used, 30 for each of the four groups: female and male chub mackerels, and female and male blue mackerels. Gonads of these mackerels were inspected to identify the phenotypic sex of each individual. Fin-clips were taken from the pectoral fins of chub and blue mackerel and used for DNA extraction. Gentra Puregene (Qiagen GmbH, Dusseldorf, Germany) and NucleoSpin Kits for Tissue (Macherey–Nagel, Duren, Germany) were used according to the manufacturer's instructions to obtain high-molecular-weight DNA. When preparing DNA for sequencing, the DNA concentration was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Species identification was conducted using a multiplex PCR kit "Saba checker-I" (SCOTS, Saga, Japan) according to the manufacturer's instructions to amplify species-specific genomic DNA regions of blue or chub mackerel in the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS1) regions.

Sequencing of male and female DNA pools

For each of the four groups described above, the same amount of DNA from each of the 30 fish was pooled and used as a sample for library construction. Library construction and sequencing were conducted at the GeT-PlaGe core facility, INRA Toulouse, France (http://get.genotoul.fr/en/). Libraries were constructed using the TruSeq nanokit (Illumina, FC-121–4001) according to the manufacturer's instructions. After the library preparation, sequencing was performed on a NovaSeq S4 lane (Illumina, San Diego, CA) using a paired-end 2 × 150 nt mode with Illumina NovaSeq Reagent Kit following the manufacturer's instructions. There were approximately 29 G base pairs of raw read for the male pool library and 51 G base pairs of raw read for the female pool library in chub mackerel, and 31 G base pairs of raw

read for the male pool library and 39 G base pairs of raw read for the female pool library in blue mackerel.

Identification of sex-specific SNVs in the de novo assemblies

Illumina paired-end reads of two *Scomber* species were assembled using DiscovarDeNovo with default parameters. Reads filtered by Sickle (ver. 1.33: ≥ Q30) from the male and female pools were remapped to a genome sequence obtained from the above-mentioned assemblies using Bowtie2 (ver.2.3.5.1). SAMtools (ver. 0.1.20) was used to convert the resulting SAM sequence alignment files to BAM formats, followed by sorting, indexing, and merging. Subsequently, BCFTOOLS (ver. 0.1.20) was used to call the variants, after which sex-specific SNVs were selected using VCFTOOLS (ver. 0.1.17, minQ 10, minDP 4, max-missing 0.2). To further screen sex-specific SNVs, the following conditions were applied to the results obtained by the VCFTOOLS: no polymorphism on the homozygous side and approximately 50% identity on the heterozygous side, > 25 reads of male and female each mapped, nonrepetitive regions, and several sex-specific SNVs residing in a neighboring region (< 500 bp) without strand bias. Integrative Genomics Viewer (ver. 2.8.2, Robinson et al. 2011) was used to confirm each condition in sex-specific SNV candidates.

Development of PCR-based sex identification with sex-specific SNVs

Specific primers were designed based on the sex-specific SNVs (Online Resource, Table S1). Then, first primer screening was performed using three male and three female samples of each species, respectively. PCR amplification was conducted in a 25-µL reaction volume containing 1× PCR Buffer II, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Ampli-*Taq* Gold DNA polymerase (Thermo Fisher Scientific, MA, USA), 50 ng of template DNA, and 1 µM of each primer. Similarly, thermal cycling conditions were one cycle of 95 ℃ for 8 min, then 35 cycles of 94 ℃ for 30 s, 55 ℃ for 15 s, 72 ℃ for 1 min, followed by a final elongation step at 72 ℃ for 7 min. Amplified PCR fragments products were then electrophoresed on a 2.0% agarose gel. PCR amplification with some primer sets was conducted using HiDi DNA polymerase (myPOLSBiotec, Konstanz, Germany) that efficiently amplifies primers that are completely matched at the 3′-end and discriminate templated DNA containing SNVs (Drum et al. 2014). By using HiDi DNA polymerase, PCR amplification was conducted in a 25-µL reaction volume tube containing 1× HiDi Buffer, 200 µM dNTPs, 2.5 U HiDi DNA polymerase, 50 ng template DNA, and 1 µM of each primer. The PCR mixtures were then cycled under the following conditions: initial denaturation at 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 15 s, 55 ℃ for 10 s, and 72 ℃ for 45 s. Verification of the PCR products was observed afterward under ultraviolet light after electrophoresis on a 2.0% agarose gel. Species identification was performed as well using a multiplex PCR kit, "Saba checker-I" (SCOTS, Saga, Japan).

Confirmation of PCR-based sex identification with various populations of mackerels

To confirm the reproducibility of this technique in various regional populations and/or year-class, chub mackerel and blue mackerel samples were collected from various areas in Japan (Fig. 1, Online Resource, Table S1). DNA samples were then extracted and identified species by Saba checker-I as described above. All samples were identified as chub mackerel or blue mackerel; there were no hybrids. DNA samples were used for the PCR-based sex identification. Identification of the phenotypic sex of each individual was performed by morphological inspection of gonads.

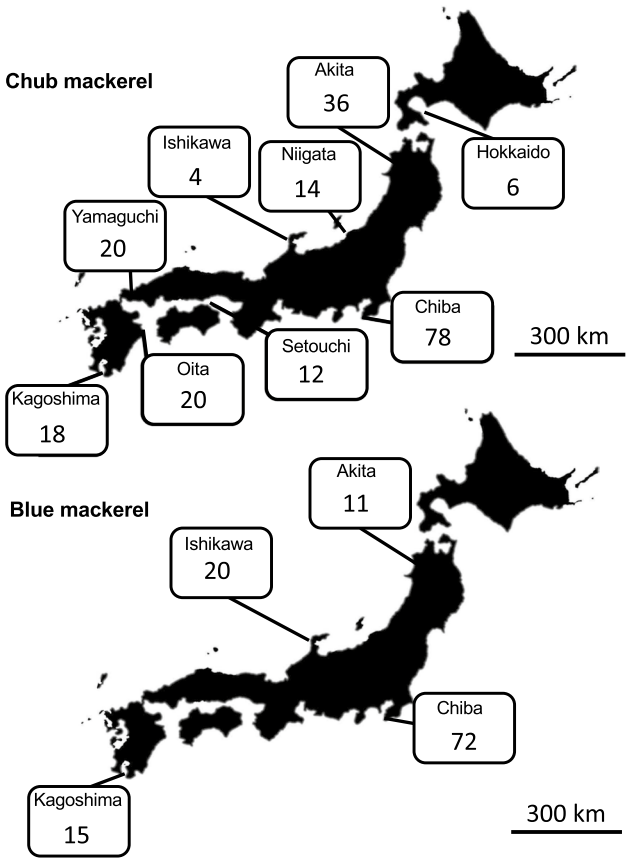

Fig. 1 Schematic figures for the sampling areas. Upper maps for chub mackerel samples, and lower maps for blue mackerel samples. The number in the boxes indicates the number of individuals collected from each area

Results

De novo assemblies and identification of sex-specific SNVs

The blue and chub mackerel sequencing resulted in 233 million and 270 million paired-end reads corresponding to 70 Gb and 80 Gb of sequence data, respectively (DRA012454). The partial de novo assemblies for blue and chub mackerel were obtained as summarized in Table 1. In total, 929,890,313 bp were assembled in 2,307,454 contigs, and 825,218,221 bp were assembled in 2,043,150 contigs that were larger than 200 bp for each species. Reads from the male and female pools were then remapped to these de novo assemblies. Similarly, sex-specific SNVs were screened as summarized in Table 2. These selected SNVs showed a significant association with males in blue mackerel while showing a significant association with females in chub mackerel. The results of this analysis fit with a XX/XY male heterogametic sex-determination system in blue mackerel and a ZZ/ZW female heterogametic system in chub mackerel.

Development of a PCR-based sex identification technique in chub and blue mackerels

Twenty-six primer sets (Online Resource, Table S2) designed based on the above-mentioned sex-specific SNVs were screened using three male and three female samples in each species. According to the results in Fig. 2, two primer sets Jpn1 and Jpn2 in chub mackerel and Aus1 and Aus2 in blue mackerel (Table 3) had 100% identification accuracy with phenotype sex. The polymorphisms between the female and male allelic sequences of sex-specific primer regions are shown in Fig. 3. Also, using the primer sets Jpn1 and Jpn 2 for the chub mackerel and Aus1 and Aus2 for blue mackerel, a medium-scale confirmation was performed with 208 samples of chub mackerel, and 118 samples of blue mackerel, respectively, collected from various areas in Japan. Consistency between the phenotypic and genetic sexes identified using PCR for each primer set is summarized in Table 4. In the chub mackerel, the primer set Jpn1 showed 98.9% identification accuracy in males, and 100% in females, while the primer set Jpn2 showed 100% identification accuracy in males, and 97.2% in females (Table 4). On the other hand, in blue mackerel, the primer set Aus1 showed 92.6% identification accuracy in males and 96.9% in females, whereas the primer set Aus2 showed 94.4% accuracy in males and 96.9% in females (Table 5). With mackerel samples collected in 2018, 2019, and 2020, respectively (Tables 4 and 5), the primer set Jpn1 in chub mackerel showed 100%, 100%, and 98.7% identification accuracy, respectively, while the primer set Jpn2 provided 99.1%, 100%, and 98.7%, identification accuracy, respectively. Similarly, in the blue mackerel

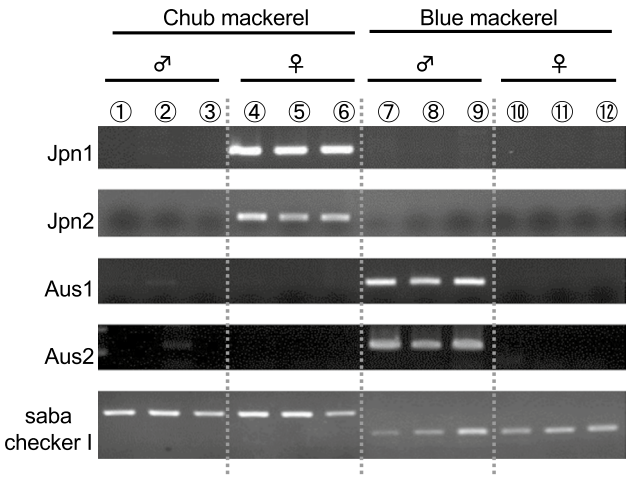

Fig. 2 Confirmation and validation of the sex-specific PCR primer sets. Female-specific fragment amplified by primer sets "Jpn1" and "Jpn2." Male-specific fragment amplified by primer sets "Aus1" and "Aus2." "Saba checker I": multiplex PCR that amplified both blue mackerel- and chub mackerel-specific regions in the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS1) region for species discrimination

Table. 1 Summary of de novo assemblies

	Chub mackerel	Blue mackerel
Number of scaffolds	20,43,150	23,07,454
Total size of scaffolds (bp)	82,52,18,221	92,98,90,313
Longest scaffold (bp)	1,06,937	1,28,293
Shortest scaffold (bp)	200	200

Table. 2 Summary of the sex-specific SNVs identification

		No. of paired reads	No. of remapped reads	No. of sex-specific SNV candidates
Blue mackerel	Female	12,94,44,376	34,784,821 (30.00%)	571
	Male	10,38,40,913	29,451,144 (30.95%)	
Chub mackerel	Female	17,14,31,237	39,898,431 (25.89%)	116
	Male	9,88,32,713	21,952,779 (24.85%)	

Table. 3 Summary of PCR primer sets

Target species	Primer set	Primer SQs		Amp. size	Enzyme
		Fwd	Rev		
Blue mackerel	Aus1	CTAGACTGAGGCTTGGGATG	ACTTTAGTCCCACAGCACGG	130 bp	HiDi
	Aus2	TGTTAGTTACAGTATAGCTT	ACGGAGGCACTTGTCTTTAC	176 bp	Ex-taq
Chub mackerel	Jpn1	TAGATGTTGTTAACTTGTGT	TACGTCTGATCCATGGTCAG	140 bp	Ex-taq
	Jpn2	ACACCATCTCACTAGGAAGA	GAGGAGGAGGTGGCGGTGGT	135 bp	HiDi

(A)

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Jpn1:
P:TAGATGTTGTTAACTTGTGT
M:TAGACGTTGTTAACTTGGGGGAGTGTGGTACAGATAATGGAGCCTCGGCTGTTGAACAAGATGAACATCTGGTTGTAGCATCATTGCTGT
F:TAGAYGTTGTTAACTTGKGKGAGTGTGGTACAGATAATGGAGCYTCGGCTGTTGAACAAGATGAACATCTGGTTGTAGCATCATTGCTGT
      *            * *                          *

P:                                CTGACCATGGATCAGACGTA
M:CCCCGACCAAAAAATCTAAATCAGGGGGTTGTGACCATGGATCAGACGTA
F:CCCCGACCAAAAAATCTAAATCAGGGGGTTSTGACCATGGATCAGACGTA
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(B)

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Jpn2:
P:ACACCATCTCACTAGGAAGA
M:ACACCATCTCACTAGGACGGGGAGACCACCCCTCAGCTTTTGTAAGGCAGGCATCTACTAGCAGAGCTTGCATTACATCTCCAACTCCCA
F:ACACCATCTCACTAGGAMGRGGAGACCACCCCTCAGCTTTTGTAAGGCAGGCATCTACTAGCAGAGCTTGCATTACATCTCCAACTCCCA
                  * *
P:                          ACCACCGCCACCTCCTCCTC
M:ACCTACAATCTCCACCTCCTCCACCGCCTCCCCCACCTCCTCCTC
F:ACCTACAATCTCCACCTCCTCCACCRCCWCCSCCACCTCCTCCTC
                            *  *  *
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(C)

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Aus1:
P:CTAGACTGAGGCTTGGGATG
M:CTAGACTGAGGCTTGGGATGGAGAGTGGGGTTTAGGCTGGCTTCTGTGATCAAGGAGGGTGCACTGTGGCTTGGGCTGGGCCAGCGGAAG
F:CTAGACTGAGGCTTGGGATGGAGAGTGGGGTTTAGGCTGGCTTCTGTGATCAAGGAGGGTGCACTGTGGCTTGGGCTGGGCCAGCGGAAG

P:                      CCGTGCTGTGGGACTAAAGT
M:GCTGGGCTGCTGGGGTCTTTMCGTGCTGTGGGACTAAAGT
F:GCTGGGCTGCTGGGGTCTTTACGTGCTGTGGGACTAAAGT
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(D)

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Aus2:
P:TGTTAGTTACAGTATAGCTT
M:TGTTAGTTACAGTATAGCTTAAATGTGATTGATGAATTTATTCTGTGAAGGCAACCTAGCCCCACAGTGGCAGATACGGAAATAAGTGGA
F:TGTTAGTTA-----TAGCTTAAATGTGATTGATGAATTTATTCTGTGAAGGCAACCTAGCCCCACAGTGGCAGATACGGAAATAAGTGGA
           *****

P:                                                                  GTAAAGACAAGTGCCTCCGTG
M:GAGGAAAACACAAATGTAAATTAGTCTAAGCAGTAAGTGTAGCTCTAGATAAACCCTGACTGGCTGTAAAGACAAGTGCCTCCGTG
F:GAGGAAAACACAAATGTAAATTAGTCTAAGCAGTAAGTGTAGCTCTAGATAAACCCTGACTGGCTGTAAAGACAAGTGCCTCCGTG
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Fig. 3 Sequence alignment of the female-specific fragment and sex-specific primer designs "Jpn 1" (**a**) and "Jpn 2" (**b**) for the chub mackerel. Sequence alignment of the male-specific fragment and sex-specific primer designs "Aus1" (**c**) and "Aus2" (**d**) for the chub mackerel. "P" indicates the sequences for the primers. "M" and "F" indicate the male allele and female allele sequences, respectively. The asterisks indicate the polymorphisms between female and male allelic sequences

Table 4 Summary of sex identification using various population samples in chub mackerel

Sampling	Sampling	No. of samples		Identity accuracy (%)*			
				Jpn1		Jpn2	
Place	Date	Male	Female	Male (%)	Female (%)	Male (%)	Female (%)
Hokkaido	2020	2	4	100.0	100.0	100.0	75.0
Akita	2020	21	15	100.0	100.0	100.0	93.3
Niigata	2020	7	7	100.0	100.0	100.0	100.0
Chiba	2018	41	37	97.6	100.0	100.0	97.3
Setouchi	2020	7	5	100.0	100.0	100.0	100.0
Ishikawa	2020	0	4	–	100.0	–	100.0
Yamaguchi	2020	7	13	100.0	100.0	100.0	100.0
Oita	2019	7	13	100.0	100.0	100.0	100.0
Kagoshima	2020	7	11	100.0	100.0	100.0	100.0
Total	–	99	109	98.9	100.0	100.0	97.2

*Concordance rate between the phenotypic and genetic sex identified by PCR-based method developed in the present study

Table 5 Summary of sex identification using various population samples in blue mackerel

Sampling	Sampling	No. of samples		Identity accuracy*			
				Aus1		Aus2	
Place	Date	Male	Female	Male (%)	Female (%)	Male (%)	Female (%)
Akita	2020	3	8	100.0	87.5	100.0	100.0
Ishikawa	2020	9	11	100.0	100.0	100.0	100.0
Chiba	2018	34	38	91.1	97.4	94.1	97.4
Kagoshima	2020	8	7	87.5	100.0	87.5	85.7
Total	–	54	64	92.6	96.9	94.4	96.9

*Concordance rate between the phenotypic and genetic sex identified by PCR-based method developed in the present study

samples collected in 2018 and 2020, the primer set Aus1 showed 95.7% and 94.4% identification accuracy, respectively, while the primer set Aus2 provided 95.7% and 95.8% identification accuracy, respectively.

Discussion

In this study, we successfully identified two female-specific DNA makers in chub mackerel, and two male-specific DNA makers in blue mackerel, respectively. Using these sex-linked DNA makers, we developed a PCR method to identify the sex of these two mackerel species with high accuracy, i.e., over 95%. The accuracy is high enough for the efficient sex management of the parental fish population in mackerel aquaculture. First, the method can be implemented using fin clipping with minimum physical stress, compared with conventional sex determination techniques conducted by observing a portion of the gonads biopsied by catheter or by measuring the sex hormones in plasma. In the conventional methods, excessive stress caused by sample collection often prevents proper gonadal maturation, especially in female broodstock, and can be an obstacle to artificial seedling production, as mackerel, in particular, are known to be vulnerable to physical stress and can die from handling (Amezawa et al. 2018). Therefore, in most cases of artificial seed production of mackerel, wild adult fish close to maturity are used since fish farmers tend to avoid maintaining parent fish for long periods. In these situations, the low-invasive sex identification methods established in this study can be effective and practical in mackerel aquaculture to manage the sex ratio of broodstock without retardation of gonadal maturation in parent fish.

Second, this method can identify the gender of mackerel at any stage of maturity with a high degree of accuracy. In contrast, it is difficult to identify the sex of immature fish using the above-mentioned conventional methods such as biopsy of the gonads or measuring plasma concentrations of sex hormones. Therefore, in mackerel, where there is no clear difference in external morphology between males and females, the method developed in this study is the only way to identify the sex of immature individuals to date. Also, since hatchery-reared breeding of mackerel has been used as broodstock recently instead of wild fish, it is also crucial to

know the sex ratio of a potential parent population as early as possible in this case. While all mackerel males spontaneously mature and produce gametes under captive conditions, not all females will produce eggs, even when hormones are administered to induce maturation. Thus, the PCR-based sex identification method developed in this study makes it possible to sort young and immature mackerels into sexes with high accuracy. Collecting the required number of female-biased parental candidates is also made possible, although identification PCR accuracy developed in this study was not 100%. Besides, using female-biased broodstock, it is possible to produce artificial seed effectively and stably to avoid wasting space, labor, and costs on excessive parental maintenance.

While the chub mackerel population is divided into the Pacific and Tsushima stocks, and blue mackerel is divided into the Pacific and East China Sea stocks, it was reported that spawning grounds for both stocks overlap in both species (Yukami et al., 2009). Since the results showed that the sex identification accuracy of PCR was consistently high in both chub and blue mackerel samples with regional differences, the two above-mentioned stocks were tested. Therefore, it is expected that the primer sets used in this study can be applied for sex prediction in most chub and blue mackerel populations distributed around Japan. This observation can be a big advantage, since some sex-linked DNA markers could be applied to only a limited pedigree (Agawa et al. 2015). Similarly, it was reported that the genetic structure of chub mackerel's population along China's coast was significantly different from that of the Japanese population (Cheng et al. 2015; Yan et al. 2015). Moreover, blue mackerel is widely distributed in tropical and subtropical waters of the Pacific Ocean from Japan to northern Australia (Collette and Nauen 1983). Therefore, further studies should confirm whether this study's methods can be applied for these genetically distant populations.

Since we have succeeded in producing feminized and masculinized mackerel by exogenous hormonal treatment (Tani et al. 2018), it was expected that mackerels potentially possess the ability for sex reversal from their genetic sex. Studies have shown that various fish exhibit environmental sex determination (ESD), where environmental factors can influence phenotypic sex during larval or juvenile development or both (Miyoshi et al. 2020). However, in this study, PCR-based molecular sex determination results with various samples showed high consistency (> 95%) between genetic and phenotypic sex in both chub mackerel and blue mackerel. These results, therefore, indicated that chub and blue mackerel rarely reversed their genetic sex in their natural environment, at least in the population caught around Japan. It should thus be considered that chub and blue mackerel possessed a rather strict genotypic sex-determination system, with no or low ESD. Another possibility is that they rarely experienced an extreme environmental condition that reversed their sex in their natural environment.

Based on the results of this study, we concluded that chub mackerel has a female heterogametic ZZ/ZW sex-determining system. This was expected as triploid chub mackerel showed a tendency for an all-female phenotype (Tani et al. 2018). Unexpectedly and interestingly, our analysis also revealed that blue mackerel has a male heterogametic (XX/XY) sex determination system. Turnovers of sex determination systems among closely related teleost species have been already reported (Devlin and Nagahama 2002, Gammerdinger et al. 2018). It is, however, surprising that these two *Scomber* species that are considered sister species with a species divergence time estimated as a few million years ago (Miya et al., 2013) possess opposite sex determination systems. In the Japanese medaka and its closely related species, a similar turnover of sex chromosomes and sex determining systems have been reported (Takehana et al. 2007, 2008). The comparative analyses in *Oryzias* species have therefore identified seven non-orthologous sex chromosomes, with six species having a heterozygous XX/XY system, and two possessing heterozygous ZZ/ZW systems, respectively (Takehana et al. 2008; Kikuchi and Hamaguchi 2013). However, whether the sex-linked SNVs of chub and blue mackerel are located on the same or different chromosomes is currently unknown as we do not have a good-quality genome reference in these species. Further studies in mackerels, with more genomic resources, should provide novel insights into the mechanisms of sex chromosome turnover in teleost. From previous studies in Scombridae, it has been reported that the Pacific bluefin tuna possess a male heterozygous XX/XY system (Suda et al. 2019), and it was speculated that eastern little tuna *Euthynnus affinis* possess a female heterozygous ZZ/ZW system (Yazawa et al. 2019). This information, therefore, suggests that there is great variety in the sex determination systems of Scombridae species.

In this study, we identified sex-linked DNA makers in chub and blue mackerel and successfully established a PCR-based sexing method employing these markers. Since this technique requires a small fin-clip and short handling time, and thus minimum physical stress compared with conventional methods such as gonad biopsy and plasma sex hormone measurement, this novel method would help maintain the health of stress-susceptible mackerels and contribute to the proper maturation and spawning of the broodstock. Also, since this method can determine sex with high accuracy regardless of the maturity stage, it can be applied to produce a broodstock population with a desired male–female ratio. Taken together, the PCR-based sexing method established in this study can be seen as a practical solution for a more stable and well-planned artificial seed supply and would help to significantly sophisticate the mackerel's aquaculture.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12562-021-01548-z.

References

Agawa Y, Iwaki M, Komiya T, Honryo T, Tamura K, Okada T, Yagishita N, Kobayashi T, Sawada Y (2015) Identification of male sex-linked DNA sequence of the cultured Pacific bluefin tuna *Thunnus orientalis*. Fish Sci 81:113–121

Amezawa K, Takeuchi Y, Yazawa R, Yoshizaki G (2018) Spawning induction of blue mackerel *Scomber australasicus* and eastern little tuna *Euthynnus affinis* by oral administration of a crude gonadotropin-releasing hormone analogue. Fish Sci 84:495–504

Chen SL, Li J, Deng SP, Tian YS, Wang QY, Zhuang ZM, Sha ZX, Xu JY (2007) Isolation of female-specific AFLP markers and molecular identification of genetic sex in half-smooth tongue sole (*Cynoglos sussemilaevis*). Mar Biotechnol 9:273–280

Cheng J, Yanagimoto T, Song N, Gao TX (2015) Population genetic structure of chub mackerel *Scomber japonicus* in the northwestern Pacific inferred from microsatellite analysis. Mol Biol Rep 42:373–382

Collette BB, Nauen CE (1983) FAO species catalogue, vol. 2. Scombrids of the world: an annotated and illustrated catalogue of tunas, mackerels, bonitos and related species known to date. FAO Fish Synop 125:137

Devlin RH, Yoshitaka N (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191–364

Drum M, Kranaster R, Ewald C, Blasczyk R, Marx A (2014) Variants of a *Thermus aquaticus* DNA polymerase with increased selectivity for applications in allele- and methylation-specific amplification. PLoS One 9:e96640

Felip A, Young WP, Wheeler PA, Thorgaard GH (2005) An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 247:35–43

Fowler BL, Buonaccorsi VP (2016) Genomic characterization of sex-identification markers in *Sebastes carnatus* and *Sebastes chrysomelas* rockfishes. Mol Ecol 25:2165–2175

Fuji K, Yoshida K, Hattori K, Ozaki A, Araki K, Okauchi M, Kubota S, Okamoto N, Sakamoto T (2010) Identification of the sex-linked locus in yellowtail, *Seriola quinqueradiata*. Aquaculture 308:S51–S55

Galindo HM, Loher T, Hauser L (2011) Genetic sex identification and the potential evolution of sex determination in Pacific halibut (*Hippoglos susstenolepis*). Mar Biotechnol 13:1027–1037

Gammerdinger WJ, Kocher TD (2018) Unusual diversity of sex chromosomes in African cichlid fishes. Genes 9:480

Hashimoto K, Osaki S, Kawamura W, Yazawa R (2019) Meat quality of frozen hybrid mackerel (*Scomber australasicus* × *Scomber japonicus*). Fish Sci 85:877–882

Kikuchi K, Hamaguchi S (2013) Novel sex-determining genes in fish and sex chromosome evolution. Dev Dyn 242:339–353

Kobayashi T, Ishibashi R, Yamamoto S, Otani S, Ueno K, Murata O (2011) Gonadal morphogenesis and sex differentiation in cultured chub mackerel, *Scomber japonicus*. Aquacult Res 42:230–239

Kovács B, Egedi S, Bártfai R, Orbán L (2000) Male-specific DNA markers from African catfish (*Clarias gariepinus)*. Genetica 110:267–276

Lee BY, Coutanceau JP, Ozouf-Costaz C, D'Cotta H, Baroiller JF, Kocher TD (2011) Genetic and physical mapping of sex-linked AFLP markers in Nile tilapia (*Oreochromis niloticus*). Mar Biotechnol 13:557–562

Li M, Xu H, Xu W, Zhou Q, Xu X, Zhu Y, Zheng W, Li W, Pang Z, Chen S (2020) Isolation of a male-specific molecular marker and development of a genetic sex identification technique in spotted knifejaw (*Oplegnathus punctatus*). Mar Biotechnol 22:467–474

Matsunaga T, Ieda R, Hosoya S, Kuroyanagi M, Suzuki S, Suetake H, Tasumi S, Suzuki Y, Miyadai T, Kikuchi K (2014) An efficient molecular technique for sexing tiger pufferfish (fugu) and the occurrence of sex reversal in a hatchery population. Fish Sci 80:933–942

Matsuyama M, Shiraishi T, Sundaray JK, Rahman A, Ohta K, Yamaguchi A (2005) Steroidogenesis in ovarian follicles of chub mackerel, *Scomber japonicus*. Zool Sci 22:101–110

Mendiola D, Yamashita Y, Matsuyama M, Alvarez P, Tanaka M (2008) *Scomber japonicus*, H. is a better candidate species for juvenile production activities than *Scomber scombrus*. L Aquacult Res 39:1122–1127

Miya M, Friedman M, Satoh TP, Takeshima H, Sado T, Iwasaki W, Yamanoue Y, Nakatani M, Mabuchi K, Inoue JG, Poulsen JY, Fukunaga T, Sato Y, Nishida M (2013) Evolutionary origin of the Scombridae (tunas and mackerels): members of a Paleogene adaptive radiation with 14 other pelagic fish families. PLoS One 8:e73535

Miyoshi K, Hattori RS, Strüssmann CA, Yokota M, Yamamoto Y (2020) Phenotypic/genotypic sex mismatches and temperature-dependent sex determination in a wild population of an Old World atherinid, the cobaltcap silverside *Hypoatherina tsurugae*. Mol Ecol 29:2349–2358

Murata O, Yamamoto S, Ishibashi R, Oka Y, Yoneshima H, Kato K, Miyashita S, Kumai H (2005) Egg development and growth of larval and juvenile cultured chub mackerel *Scomber japonicus* (Perciformes: Scombridae) in a captive spawning experiment. Aquacult Sci 53:319–324 (**(In Japanese with English abstract)**)

Ohga H, Ito K, Matsumori K, Kimura R, Ohta K, Matsuyama M (2020) Leptin stimulates gonadotropin release and ovarian development in marine teleost chub mackerel. Gen Comp Endocrinol 292:113442

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Jill P (2011) Integrative genomics viewer. Nat Biotechnol 29:24–26

Schlötterer C, Tobler R, Kofler R, Nolte V (2014) Sequencing pools of individuals—mining genome-wide polymorphism data without big funding. Nat Rev Genet 15:749–763

Shiraishi T, Ohta K, Yamaguchi A, Yoda M, Chuda H (2005) Reproductive parameters of the chub mackerel *Scomber japonicus* estimated from human chorionic gonadotropin-induced final oocyte maturation and ovulation in captivity. Fish Sci 71:531–542

Suda A, Nishiki I, Iwasaki Y, Matsuura A, Akita T, Suzuki N, Fujiwara A (2019) Improvement of the Pacific bluefin tuna (*Thunnus orientalis*) reference genome and development of male-specific DNA markers. Sci Rep 9:14450

Takehana Y, Naruse K, Hamaguchi S, Sakaizumi M (2007) Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena*. Chromosoma 116:463–470

Takehana Y, Hamaguchi S, Sakaizumi M (2008) Different origins of ZZ/ZW sex chromosomes in closely related medaka fishes, *Oryzias javanicus* and *O. hubbsi*. Chromosome Res 16:801–811

Tani R, Yazawa R, Yoshizaki G (2018) Production of triploid chub mackerel and development of feminization and masculinization technique and in scombrids. Unpublished manuscript, Tokyo University of Marine Science and Technology, Japan

Xu D, Lou B, Xu H, Li S, Geng Z (2013) Isolation and characterization of male-specific DNA markers in the rock bream *Oplegnathus fasciatus*. Mar Biotechnol 15:221–229

Yan S, Catanese G, Brown CL, Wang M, Yang C, Yang T (2015) Phylogeographic study on the chub mackerel (*Scomber japonicus*) in the northwestern Pacific indicates the Late Pleistocene population isolation. Mar Ecol 36:753–765

Yazawa R, Takeuchi Y, Machida Y, Amezawa K, Kabeya N, Tani R, Kawamura W, Yoshizaki G (2019) Production of triploid eastern little tuna, *Euthynnus affinis* (Cantor, 1849). Aquacult Res 50:1422–1430

Yukami R, Ohshimo S, Yoda M, Hiyama Y (2009) Estimation of the spawning grounds of chub mackerel *Scomber japonicus* and spotted mackerel *Scomber australasicus* in the East China Sea based on catch statistics and biometric data. Fish Sci 75:167–174